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TITLE: Novel Functions of EZH2 in Triple Negative Breast Cancer: Translation in to New Biomarker and Treatment Strategies.

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13. SUPPLEMENTARY NOTES		
14. ABSTRACT In our first year of funding we have been able to identify 68 TNBC tissue samples from African women, and 60 TNBC from White women. All blocks were organized and paraffin embedded sections were stained with hematoxylin and eosin (H&E) and histologically evaluated. We successfully developed high density tissue microarrays with these cases. Furthermore, we have selected and optimized the antibodies against EZH2, p38, and H3K27me3 to use for immunohistochemical staining of these tissue microarrays. We are currently working on optimizing anti- AKT1. We have been able to characterize the protein-protein interactions (PPIs) between EZH2 and p38 α and with AKT1. We successfully determined the kinetic and binding affinity of these PPIs using recombinant full length proteins. These results confirmed the direct interactions between EZH2 and p38 α and AKT1 as well as the co-immunoprecipitations studies which showed that EZH2 binds to p38 α in both the nucleus and the cytoplasm of MDA-MB-231 cells. We will continue with biochemical and biophysical studies towards mapping the binding site and identifying the key residues essential for these PPIs.		
15. SUBJECT TERMS Triple negative breast cancer, EZH2, disparities		

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1. INTRODUCTION

This is the first annual report for a project that aims to understand the non-canonical functions of EZH2 as a determinant of breast cancer invasion and metastasis, and to elucidate the relevance of cytoplasmic expression of EZH2. EZH2 (Enhancer of Zeste Homolog 2), a Polycomb group protein presumed to function in controlling the transcriptional memory of a cell [1], is up-regulated during progression from ductal carcinoma in situ, the precursor of invasive carcinoma, to invasive carcinoma and distant metastasis [2]. Furthermore, EZH2 protein is over-expressed in 55% of invasive breast carcinomas, and is significantly associated with poorly differentiated tumors [2-4]. Our laboratory has previously shown that EZH2 is a powerful independent prognostic biomarker in breast cancer, providing outcome information above and beyond conventional prognosticators used in the clinical setting [2]. We have also demonstrated by Kaplan-Meier analysis that tumors with high EZH2 expression had a worse disease free and overall survival than tumors with low EZH2 expression. Our recent studies support that EZH2 is expressed in the cytoplasm in aggressive breast carcinomas from Ghanaian women [5]. Furthermore, we have found that EZH2 binds to p38 and AKT1 in breast cancer cells, leading to their methylation. These data led us to hypothesize that EZH2 is expressed in the cytoplasm of a subset of TNBC tumors where it methylates and activates p38 and AKT1 leading to metastasis. We further hypothesize that detection of cytoplasmic EZH2, phosphorylated p38 and/or phosphorylated AKT1 proteins may identify TNBC tumors with more aggressive behavior and heightened metastasis. As I will illustrate below, we have made progress in this first year of funding.

2. **KEYWORDS:** triple negative breast cancer, EZH2, African, health disparities, protein-protein interactions.

3. ACCOMPLISHMENTS

Below are brief descriptions of key accomplishments according to the approved statement of work for Year 1.

Aim 1: To investigate whether the concordant expression of cytoplasmic EZH2, phospho-p38 and/or phospho-AKT1 in tissue samples identifies TNBC with high metastatic ability in African, AA, and White women.

- **Task 1:** To test the pattern of expression of EZH2, phosphorylated p38, phosphorylated AKT1, and H3K27me3 proteins in invasive carcinomas of women from different races (Years 1-1.5).
- Task 2 takes place on years 2-3.

During this first year we were able to identify tissue blocks of Ghanaian and White patients' breast carcinomas and characterized them histologically. We also developed several high density tissue microarrays to facilitate the immunohistochemical studies. We have also selected the appropriate antibodies against all of the proteins mentioned above, and optimized the conditions for immunohistochemistry of human breast tissue samples. We are currently performing immunostains and will be interpreting them during our second year of funding, as outlined in the statement of work. **Figure 1** shows representative images of our work for Task 1.

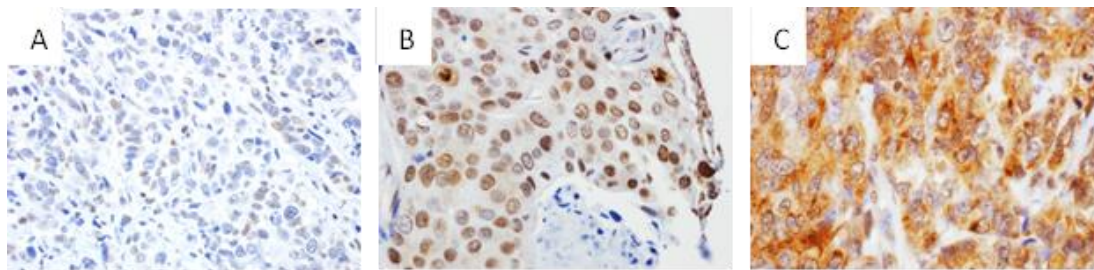


Figure 1. Representative images of invasive carcinomas from Ghanaian patients. **A.** negative EZH2

B. nuclear EZH2, and **C.** cytoplasmic EZH2.

Aim 2: Biochemical and biophysical characterization of the EZH2 interaction with p38 and AKT1 proteins and mapping EZH2 binding site that interacts with p38 and AKT1.

- Task 3: Determine the biochemical features of the EZH2 binding to p38 and AKT1 (Years 1-2).

During the first year we were able to quantitatively determine the binding affinities of EZH2 protein-protein interactions involved with p38 and AKT1. For this purpose we used full length recombinant proteins. To determine the direct binding and kinetic of the interactions we used bio-layer interferometry method. For this purpose EZH2 protein was successfully biotinylated and immobilized on streptavidin sensors and used for testing the binding of p38 and AKT1 and determine the kinetics of these PPIs. The obtained results showed that EZH2 has significantly higher binding affinity to p38 with K_D of 24 nM (**Figure 2**). Importantly, the obtained results confirmed the co-immunoprecipitation studies which detected endogenous EZH2-p38 interaction in the nucleus and in the cytoplasm of MDA-MB-231 TNBC cells. In contrast, EZH2 binds AKT1 with 14 fold decreased binding affinity ($K_D = 326$ nM).

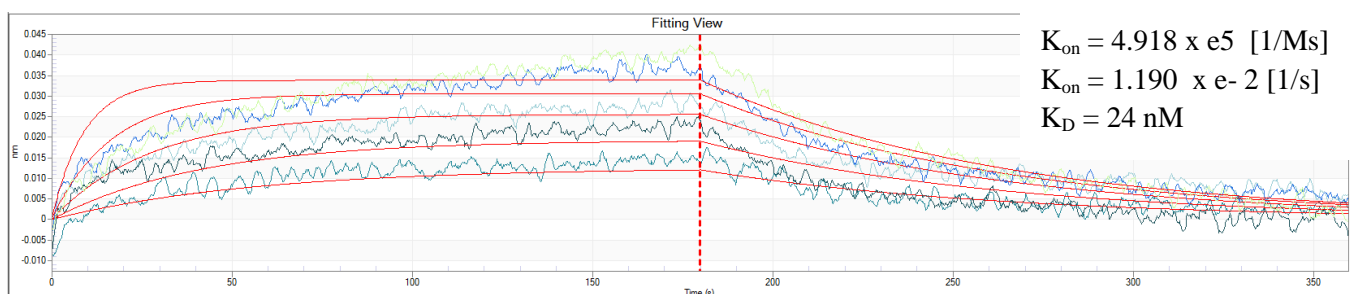


Figure 2. Binding and kinetic data of the PPIs between EZH2 and p38 determined by bio-layer interferometry method. EZH2 protein was immobilized on the streptavidine sensors and p38 was tested in following concentrations: 12 nM, 25 nM, 50 nM, 100 nM and 200 nM. The binding curves were fitted using 1:1 Langmuir model.

Because of the stronger binding affinity of EZH2 protein to p38 in the next year we will focus on understanding the interaction sites between these two proteins. This is further supported by the importance of p38 signaling as a major promoter of breast tumorigenesis. We will prepare sets of recombinant protein deletion variants to determine the minimum region in EZH2 and p38T1 required for these PPIs. The binding affinity constants will be confirmed by applying surface plasmon resonance analysis. Once this is accomplished, we will generate a series of point mutations that will be tested in binding assays to identify the key residues involved in the interaction and map the minimum fragments required for high affinity interaction. Based on these results we will synthesize peptides to further

confirm the interactions and through applying alanine scanning mutagenesis strategy we will be able to determine the nature of these PPIs, electrostatic and/or hydrophobic interactions.

- **Task 4:** To test the binding of EZH2 to p38 and AKT1 in breast cancer cells and in cancer cells freshly isolated from patients with TNBC (Year 1-1.5).

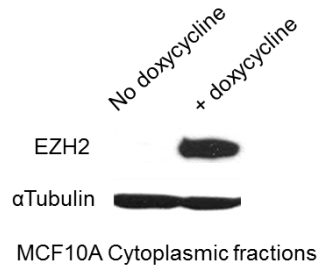
During this first year of funding we tested the expression of EZH2 in a panel of breast cancer cells derived TNBC. As shown in **Figure 3**, EZH2 expression is high in TNBC cells including MDA-MB-231 and in T4 cells, derived from a fresh TNBC tumor sample that we isolated in our laboratory.

Figure 3. We isolated breast cancer cells from fresh breast cancer tissues from 2 patients with TNBC, and a lymph node metastasis from patient 2. EZH2 is elevated in all samples. EZH2 is also elevated in TNBC cell line MDA-MB-231.



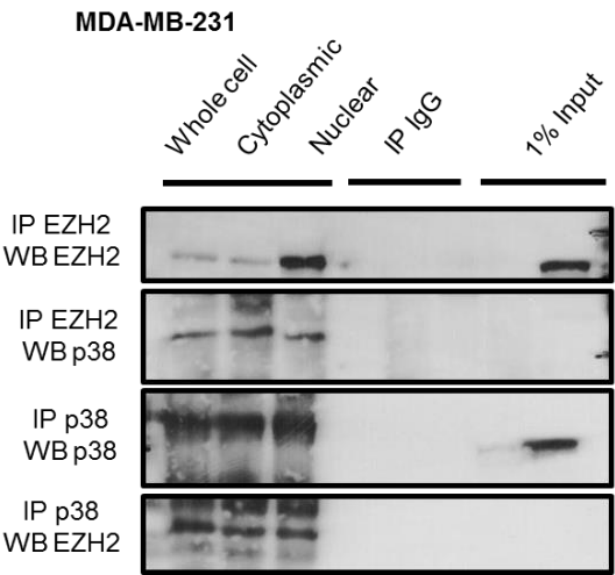
Our lab has demonstrated that EZH2 overexpression in MCF10A cells leads to increased invasion and migration. We noted also that EZH2 overexpression leads to increased EZH2 levels in the cytoplasmic-enriched fractions (**Figure 4**).

Figure 4. Using a doxycycline inducible system in MCF10A cells that we have previously developed in the lab, we demonstrated that upon 24 hours of doxycycline treatment, EZH2 is expressed in the cytoplasm of breast cells.



To begin to test the hypothesis that EZH2 exerts functions in the cytoplasm of breast cancer cells, we performed co-immunoprecipitations of cytoplasmic and nuclear enriched fractions of MDA-MB-231 cells. **Figure 5** shows that EZH2 binds to p38 in both the nucleus and the cytoplasm of these cells. These data provides the foundation to test patient-derived breast cancer cells in ongoing experiments in our laboratory. We are also testing the binding between EZH2 and AKT1 proteins in these cell lines and patient-derived TNBC cells.

Figure 5. We extracted nuclear and cytoplasmic fractions of MDA-MB-231 and detected endogenous EZH2-p38 interaction in the nucleus and in the cytoplasm of MDA-MB-231 TNBC cells.



Aim 3: To elucidate the relevance of EZH2-mediated binding on TNBC invasion and metastasis *in vivo* and *in vitro*. These experiments are planned for years 2 and 3.

References

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2. Kleer, C.G., et al., *EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells*. Proc Natl Acad Sci U S A, 2003. **100**(20): p. 11606-11.
3. Bachmann, I.M., et al., *EZH2 expression is associated with high proliferation rate and aggressive tumor subgroups in cutaneous melanoma and cancers of the endometrium, prostate, and breast*. J Clin Oncol, 2006. **24**(2): p. 268-73.
4. Collett, K., et al., *Expression of enhancer of zeste homologue 2 is significantly associated with increased tumor cell proliferation and is a marker of aggressive breast cancer*. Clin Cancer Res, 2006. **12**(4): p. 1168-74.
5. Pang, J., et al., *Invasive breast carcinomas in Ghana: high frequency of high grade, basal-like histology and high EZH2 expression*. Breast Cancer Res Treat, 2012. **135**(1): p. 59-66.

Thus, the key research accomplishments in this year of work are:

- Optimized EZH2, p38, phospho-p38, AKT1, phospho-AKT1 antibodies to use in immunohistochemistry of human tissues.
- Detected EZH2 in the cytoplasm of human breast cancers from Ghanaian women.
- Detected EZH2 in the cytoplasm of TNBC cell lines.
- Isolated TNBC cells from fresh samples of human breast carcinomas, and from one lymph node metastasis, and detected high EZH2 protein expression, comparable to MDA-MB-231 cells.
- Developed high density tissue microarrays with breast cancers from Ghana to use in our biomarker development studies.
- Prepare tools to run direct binding studies including biotinylated EZH2 and immobilizing SSA sensors
- Determined the kinetic parameters, k_{on} and k_{off} , of the PPIs between EZH2 and p38 and with AKT1.
- Determined the K_D of investigated PPIs: EZH2 – p38 and EZH2-AKT1.

We are poised to carry out the biomarker studies proposed in our application, which may provide the foundation for a useful test to detect aggressive behavior in TNBC. We plan to continue our project as approved by the DOD.

This project has given us the opportunity to train several undergraduate and graduate students in the laboratory. It gave them the opportunity to learn about the histopathology of human breast cancer, and the pathologist approach to making a diagnosis and classification of tumors. They also learned how to perform molecular biology techniques and immunohistochemistry. We gained from this experience by having time to teach and discuss important aspects of breast cancer histology and health disparities to my lab member during this year. We have also attended the AACR meetings in 2015 and 2016 and presented the work. Attending the AACR meeting was extremely beneficial as we participated in minisymposium, plenary lectures, and poster presentations which broadened our knowledge and understanding of breast cancer development and progression. We disseminated our results through poster presentations at AACR and University of Michigan meetings.

4. IMPACT

Our project during this year has been impactful in two aspects:

1. By demonstrating that EZH2 is expressed in the cytoplasm of TNBC and that it interacts with MAPK p38 protein. This is a novel idea that has not been studied, and, together with our future studies will represent a major paradigm shift in our understanding of how EZH2 drives TNBC, and may provide new biomarkers of aggressive breast cancer, and targeted therapies.
2. For the first time we have determined the binding affinity and kinetics of the protein-protein interactions between EZH2 and p38 as well as with AKT1. The obtained results showed that EZH2 has higher binding affinity to p38 in comparison with AKT1, which will contribute to understanding of how EZH2 drives TNBC, and may provide new biomarkers of aggressive breast cancer, and targeted therapies. Our future studies towards mapping the interaction site, the potency and the nature of these PPIs, will give us insights as to whether these PPIs are “druggable” target and the feasibility of development of small molecules targeting the binding site of EZH2 to p38 and/or to AKT1.

5. CHANGES/PROBLEMS

No problems to report. No changes to the original aims and tasks.

6. PRODUCTS

Talha Anwar, Heather Moore, Maria E. Gonzalez, Celina Kleer. Identification of a novel interaction between EZH2 and p38 in triple negative breast cancer. Pathology Research Symposium, University of Michigan, Ann Arbor, MI, February 2015. Poster presentation.

Talha Anwar, Maria E. Gonzalez, Wei Huang, Celina Kleer: Noncanonical functions of EZH2 in triple negative breast cancers. AACR meeting, April 2015. Poster presentation.

Talha Anwar, Caroline Arellano-Garcia, Maria E. Gonzalez, Celina Kleer. Phosphorylation regulates EZH2 neoplastic functions in triple-negative breast cancer. AACR meeting, April 2016. Poster presentation. Awarded a prize from the **AACR Triple Negative Breast Cancer Foundation Award**.

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

Celina G. Kleer, M.D.

Principal investigator (initiating PI)

ORCID ID: 0000-0001-8195-185X

Nearest person month worked: 3

Contribution to project: planned direction of project to follow SOW, designed experiments and guided the student, analyzed tissue samples, oversaw construction of TMAs, analyzed results, assisted with writing of abstracts and posters.

Zaneta Nikolovska-Coleska, Ph.D.

Partnering PI

Nearest person month worked: 1

Contribution to project: planned direction of project to follow SOW, designed experiments and guided the postdoctoral fellow, analyzed and summarizing the obtained results.

Talha Anwar, B.S.

Graduate Student

Nearest person month worked; 6

Contribution to project: read the literature, designed experiments and discussed with PI, carried out experiments and analyzed the results, wrote abstracts and posters.

Lei Miao, Ph.D.

Postdoctoral Fellow

Nearest person month worked: 3

Contribution to project: read the literature, designed experiments and discussed with PI, carried out experiments and analyzed the results.

8. SPECIAL REPORTING REQUIREMENTS

As this is a collaborative award, we are providing one report for both the initiating and partnering PIs, as requested by the DOD.

9. APPENDIX, not necessary.